The Journal of Immunology

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J Immunol published online 6 March 2020
http://www.jimmunol.org/content/early/2020/03/05/jimmunol.1900315

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published March 6, 2020, doi:10.4049/jimmunol.1900315

Induction of IκBζ Augments Cytokine and Chemokine Production by IL-33 in Mast Cells

Hiromi Ohto-Ozaki,* Morisada Hayakawa,*,† Nobuhiko Kamoshita,*,† Takashi Maruyama,‡ Shin-ichi Tominaga,*,§ and Tsukasa Ohmori*,†

IκBζ (encoded by the *Nfkbiz*) is a member of the nuclear IκB family, which is involved in the expression of secondary response genes based on signals from TLR or IL-1R. ST2L, an IL-33R, is a member of the IL-1R family and abundantly expressed in tissue-resident immune cells, such as mast cells and innate lymphoid cells; however, its downstream signaling pathway remains unelucidated. In this study, we examined the role of IκBζ in ST2L-mediated cytokine and chemokine production in mast cells. Murine bone marrow cells were differentiated ex vivo into bone marrow–derived mast cells (BMMCs). The treatment of BMMCs with IL-33 transiently induced robust IκBζ expression. Of the 40 cytokines and chemokines examined using a cytokine and chemokine array, the concentrations of IL-6, IL-13, CCL2, CCL3, and TNF-α in the supernatant were augmented by IL-33. The deletion of IκBζ in BMMCs resulted in a significant reduction of the production of these mediators and the expression of their mRNA. NF-κB p50 but not p65 translocated to the nucleus by IL-33 and was not affected by the deletion of IκBζ. However, induction of IκBζ and the resultant cytokine and chemokine productions were significantly inhibited by pretreatment with an NF-κB inhibitor. The deletion of IκBζ did not affect the phosphorylation of ERK, p38 MAPK, or JNK by IL-33, and the treatment with inhibitors of these mitogen-activated kinases failed to abolish the expression of *Nfkbiz*. Our findings suggest that IκBζ augments IL-33–dependent cytokine and chemokine production in BMMCs through the action of NF-κB. *The Journal of Immunology*, 2020, 204: 000–000.

Interleukin-33 is a nuclear cytokine produced by structural cells, such as endothelial cells, epithelial cells, and fibroblasts (1, 2). IL-33 belongs to the IL-1 superfamily and is released as an alarm signal (alarmin) upon cell injury (1, 2). IL-33 binds to a specific receptor, ST2L, which is a member of the of the IL-1R superfamily. We originally identified the soluble form of this receptor, ST2, in fibroblasts stimulated with serum (3, 4) and then cloned the transmembrane form ST2L as an orphan receptor (5). It is now known that ST2L forms a heterodimer with the IL-1R accessory protein (IL-1RAcP) to elicit immune responses through IL-33 (1, 6). ST2L is abundantly expressed on mast cells, group 2 innate lymphoid cells (ILC2s), NK cells, and Th2 lymphocytes (7). IL-33 alerts these immune cells and so augments inflammation through its interaction with ST2L (7, 8). As a result, the IL-33/ST2L axis is a critical modulator of the immune system and is involved in the pathophysiology of immune diseases.

Mast cells are potent tissue-resident immune defense cells that are distributed in barrier tissues such as the skin and mucosa (9, 10). The activation of mast cells is known to be involved in many pathophysiological settings (e.g., vasodilation, angiogenesis, and innate and adaptive immune responses) (11). The abundant expression of ST2L on mast cells suggests important roles for IL-33 in these processes. Indeed, IL-33 can control a variety of cellular functions in mast cells, including adhesion, maturation, and cytokine production (6). Notably, mast cells stimulated with IL-33 are recognized to be a major driver of the high levels of IL-6 and IL-13 that further exacerbate allergic-type immune responses (12). Understanding the details of regulatory mechanisms whereby IL-33 activates mast cells may be crucial to identifying new therapeutic targets for allergic diseases.

IL-33 mediates a complex downstream signaling cascade through its interaction with ST2L/IL-1RAcP (1, 6). The cytoplasmic domain of ST2L/IL-1RAcP, also called the Toll/IL-1R (TIR) domain, shares structural similarities with those of IL-1R, IL-18R, IL-36R, and TLRs (11). The TIR domain acts as a scaffold to recruit signaling molecules, including MyD88 adapter protein, the IL-1R–associated kinase (IRAK) 1, IRAK4, and TNFR-associated factor 6 (TRAF6) (13, 14). The signaling complex ultimately activates NF-κB and MAPKs and reportedly regulates cytokine production (12). However, the cellular and molecular mechanisms proceeding downstream of IL-33 that exacerbate the immune reaction of mast cells have not been fully elucidated.

IκBζ, is an atypical member of the nuclear IκBζ family that is encoded by the *Nfkbiz* gene (15). IκBζ amplifies the expression of secondary response genes to enhance cytokine production by TLR/IL-1R (16). In this study, we focused on the role of IκBζ in the production of cytokines by mast cells stimulated with IL-33.

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Received for publication March 14, 2019. Accepted for publication February 7, 2020.

This work was supported in part by research support from Bayer, Daiichi Sankyo, and CSL Behring. T.O. received research grant support from Bayer, Daiichi Sankyo, and CSL Behring for this study.

H.O.-O. designed the study, performed the experiments, analyzed data, and wrote the manuscript. M.H. designed the study and revised the paper. N.K. revised the manuscript. T.M. provided gene-deficient mice and revised the manuscript. S.-i.T. revised the manuscript. T.O. designed the study, analyzed the data, and wrote the manuscript. All authors approved the final version of the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMMC, bone marrow–derived mast cell; c-Kit, CD117; *Hprt1*, hypoxanthine-guanine phosphoribosyltransferase 1; IL-1RAcP, IL-1 receptor accessory protein; IRAK, IL-1R–associated kinase; p38, p38 MAPK; poly(I:C), polyinosinic-polycytidylic acid; TIR, Toll/IL-1 receptor.

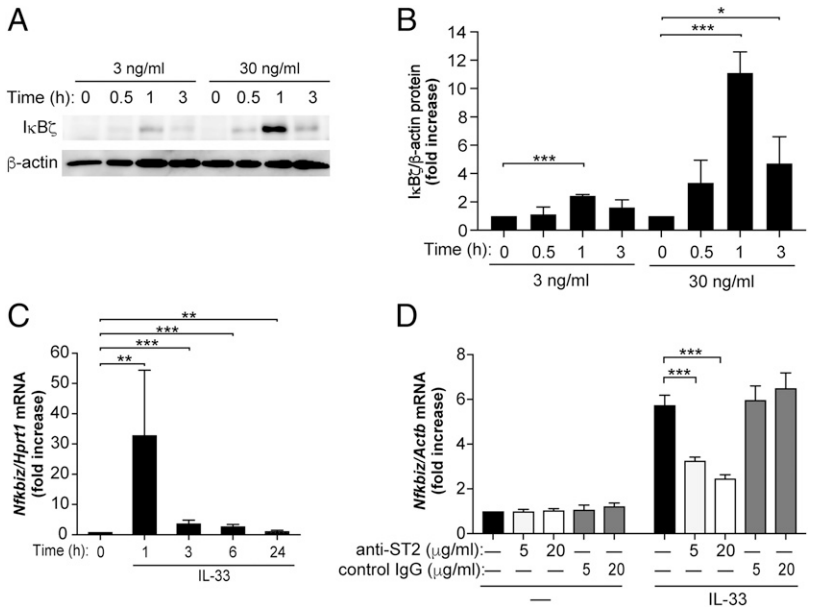

FIGURE 1. Induction of IκBζ by IL-33 in mast cells. (**A** and **B**) BMMCs were stimulated with 3 or 30 ng/ml IL-33 for the indicated time. (A) Protein expression of IκBζ was assessed using immunoblotting (upper panel). β-actin was used as an internal control (lower panel). Data are representative of three experiments. (B) The expression of IκBζ was quantified using ImageJ software and expressed as a fold increase in protein expression. Band intensities were normalized to β-actin. Data were expressed as the mean ± SD ($n = 3$). *$p < 0.05$, ***$p < 0.001$. (**C**) mRNA induction of *Nfkbiz* by 3 ng/ml IL-33 was assessed using real time RT-PCR and expressed as the fold increase in the *Nfkbiz/Hprt1* ratio. Values are mean ± SD ($n = 7$). **$p < 0.01$, ***$p < 0.001$. (**D**) The BMMCs were preincubated with 5 or 20 μg/ml control IgG or anti-ST2 mAb for 1 h and then stimulated with 3 ng/ml IL-33 for 3 h. *Nfkbiz* mRNA expression was assessed by real time RT-PCR and expressed as the fold increase in the *Nfkbiz/Actb* ratio (black bar, no-pretreatment; white bar, treatment with anti-ST2 Ab; gray bar, treatment with control Ab). The data were expressed as mean ± SD ($n = 3$). ***$p < 0.001$.

Materials and Methods

Reagents and Abs

The following reagents were obtained from the specified suppliers: recombinant mouse IL-33, recombinant mouse IL-3, purified anti-mouse CD16/CD32 mAb, and allophycocyanin-labeled anti-mouse IL-33Rα (IL-1RL1, ST2L) rat mAb (BioLegend, San Diego, CA); anti-mouse IκBζ rabbit polyclonal Ab (Bethyl Laboratories, Montgomery, TX); anti-rabbit IgG conjugated with HRP (Jackson ImmunoResearch, West Grove, PA); anti–β-actin mAb and anti-IκBα rabbit polyclonal Ab (Santa Cruz Biotechnology, Dallas, TX); rat IgG (Sigma-Aldrich, Saint Louis, MO); anti-mouse IgG conjugated with HRP (Bio-Rad Laboratories,

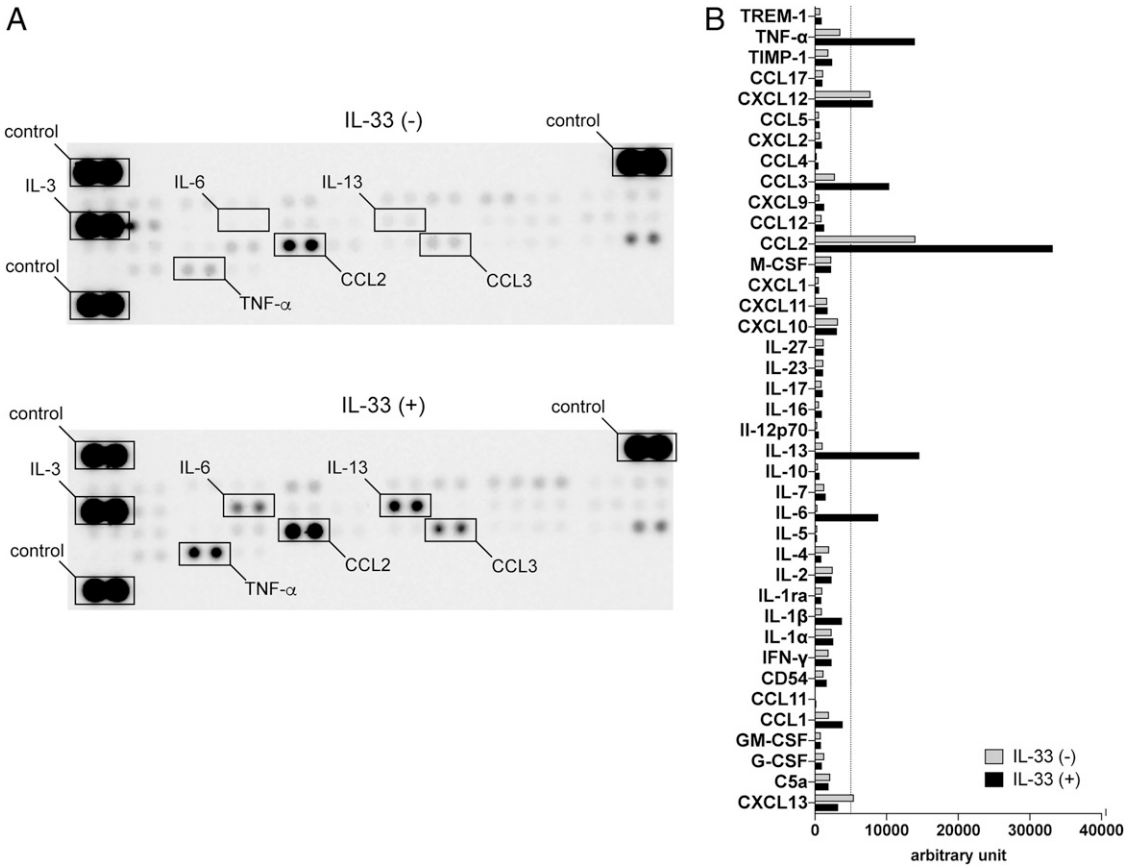

FIGURE 2. Cytokine and chemokine profile produced by mast cells stimulated with IL-33. BMMCs were stimulated with or without 3 ng/ml IL-33 for 3 h. The cytokine and chemokine levels in the supernatant were assessed using a Mouse Cytokine Array, Panel A (R&D Systems). (**A**) Representative blotting of cytokine panels using supernatant produced without (upper panel) or with IL-33 (lower panel) treatment for 3 h. Box represents the location of spots for selected cytokines, IL-3, a cytokine used for differentiation of BMMCs, and control spots. (**B**) The relative expression of each cytokine was quantified from pixel densities using an ImageQuant LAS4000 (GE Healthcare) (gray bar, no treatment; black bar, treatment with IL-33). Dotted line indicates expression cutoff level. Data are representative of two independent experiments.

Hercules, CA); anti-mouse ST2/IL-1R rat mAb and Mouse IL-13 ELISA Kit (R&D Systems, Minneapolis, MN); PE-labeled anti-CD117 (c-Kit) mAb and FITC-labeled anti-FcεRI mAb (Tonbo Biosciences, San Diego, CA); Mouse IL-6 ELISA Kit, Mouse CCL3 ELISA Kit, Mouse CCL2 ELISA Kit, and Mouse TNF-α ELISA Kit (Thermo Fisher Scientific, Waltham, MA); BAY 11-7082 (Cayman Chemical, Ann Arbor, MI); anti–p38 MAPK (p38) rabbit mAb, anti–phospho-p38 rabbit mAb, anti-ERK rabbit polyclonal Ab, anti–phospho-ERK rabbit mAb, anti-JNK rabbit mAb, anti–phospho-JNK rabbit mAb, anti–NF-κB1 p105/p50 rabbit mAb (D4P4D), anti–NF-κB1 p65 rabbit mAb (C22B4), anti–phospho-Stat5 (Tyr694) rabbit polyclonal Ab, anti-Stat5 Rabbit mAb (D2O6Y), and U0126 (Cell Signaling Technology, Danvers, MA); SP600125 (FUJIFILM Wako Pure Chemical, Osaka, Japan); mouse recombinant stem cell factor (Miltenyi Biotec, Sunnyvale, CA); and SB203580 (Enzo Life Sciences, Farmingdale, NY).

Ethics approval

The Institutional Animal Care and Concern Committee of Jichi Medical University approved all animal procedures, and animal care was conducted in accordance with the committee's guidelines.

Generation of Nfkbiz conditional-deficient mice

Mice containing *loxP*-flanked *Nfkbiz*$^{fl/fl}$ were previously generated by gene targeting (17). Haploinsufficiency of the *Nfkbiz* allele (*Nfkbiz*$^{+/-}$) was generated by breeding *Nfkbiz*$^{fl/fl}$ animals with *CAG-Cre* transgenic mice (bank number RBRC01828; Riken BioResource Research Center, Ibaraki, Japan) (18). The deletion of both *Nfkbiz* alleles from hematopoietic cells was achieved by breeding *Nfkbiz*$^{fl/fl}$ animals with *Mx1-Cre* transgenic mice (bank number nbio227; Health Science Research Resources, Osaka, Japan) (19). Induction of Cre in hematopoietic cells was achieved by peritoneal injection of polyinosinic-polycytidylic acid [poly(I:C); 300 μg/body, four times] to *Mx1-Cre;Nfkbiz*$^{fl/fl}$ and littermate control (*Nfkbiz*$^{fl/fl}$). After 4–6 wk, IκBζ alleles in the genomic DNA of WBCs were assessed by PCR using the primers shown in Supplemental Table I.

Preparation of bone marrow–derived mast cells

Bone marrow cells were isolated from tibia and femur of mice. Bone marrow cells were cultured in RPMI 1640 containing 10% FBS, 1× MEM Nonessential Amino Acids Solution, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 5 ng/ml IL-3 to differentiate them into the mast cells. The differentiation into mast cells was assessed using flow cytometry. Before experimentation, we confirmed the expression of c-Kit and FcεRI in more than 95% of the cells.

Preparation of cytoplasmic and nuclear fractions

The cells were resuspended in ice-cold 10 mM HEPES buffer (pH 7.9) containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF, and 1× cOmplete Protease Inhibitor (Roche Diagnostics, Basel, Switzerland) and incubated at 4°C for 15 min. After the addition of NP-40 (final concentration 0.625%), the lysate was centrifugated at 15,000 × *g* for 1 min. The supernatant was then recovered as the cytoplasmic fraction. The resultant pellet was resuspended in nuclear lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 20 mM NaF, 1 mM Na_3VO_4,

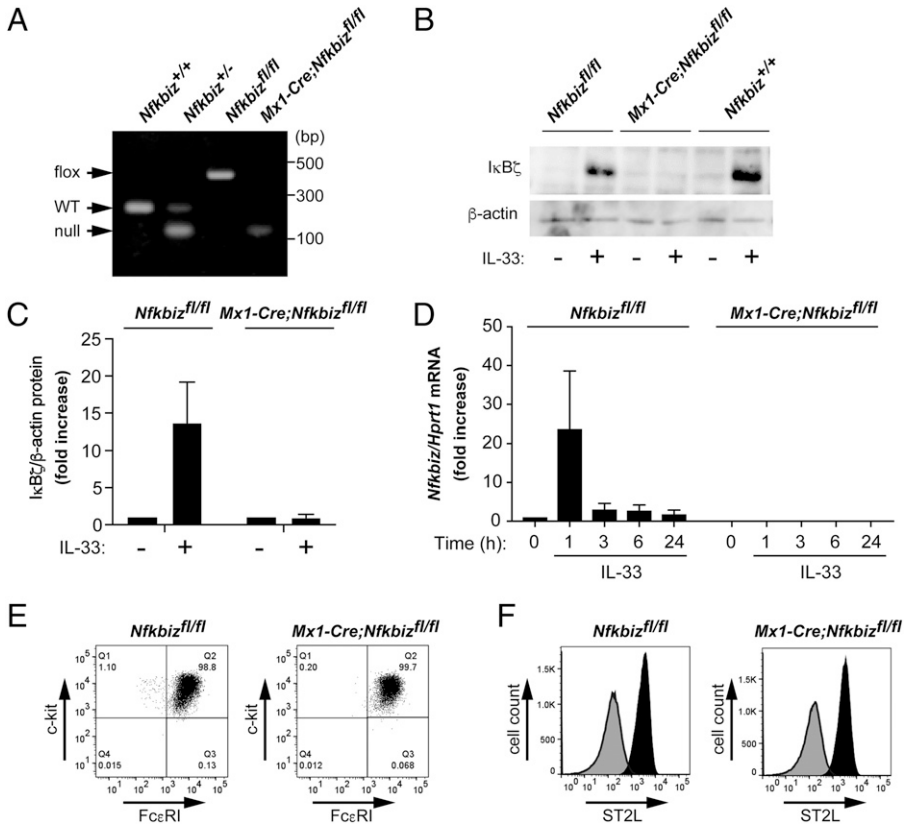

FIGURE 3. Generation of IκBζ-deficient mast cells. (**A**) DNA genotyping of WBCs obtained from wild-type C57BL6 *Nfkbiz*$^{+/+}$, *Nfkbi*$^{-/+}$, *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) was assessed by PCR. Arrows indicate the flox allele, wild-type allele, and null allele. Data are representative of three experiments. (**B** and **C**) The BMMCs obtained from *Nfkbiz*$^{fl/fl}$, *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C), or from C57BL/6 *Nfkbiz*$^{+/+}$ mice were treated with or without 30 ng/ml IL-33 for 1 h. (B) The protein expression of IκBζ was assessed using immunoblotting (upper panel). β-actin was used as an internal control (lower panel). Data are representative of three experiments. (C) The expression of IκBζ by IL-33 in the BMMCs obtained from *Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) were quantified by ImageJ software and expressed as a fold increase in protein expression. Band intensities were normalized to β-actin. Data were expressed as the mean ± SD ($n = 3$). (**D**) Induction of *Nfkbiz* mRNA by 3 ng/ml IL-33 in BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) was assessed by real-time RT-PCR and expressed as the fold increase in the *Nfkbiz*/*Hprt1* ratio (left, *Nfkbiz*$^{fl/fl}$; right, *Mx1-Cre;Nfkbiz*$^{fl/fl}$). Values are show as mean ± SD ($n = 5$). Statistical significance was ND because all data of *Mx1-Cre;Nfkbiz*$^{fl/fl}$ were undetectable. (**E**) Expression of c-Kit and FcεRI in BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice was assessed by flow cytometry. The plots represent the degree of c-Kit (vertical) and FcεRI (horizontal) expression. (**F**) Expression of ST2L on BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice was assessed by flow cytometry (gray, control; black, anti-ST2 Ab).

1× cOmplete Protease Inhibitor) and incubated at 100°C for 10 min. After centrifugation at 15,000 × *g* for 10 min, the supernatant was recovered as the nuclear fraction.

Immunoblotting

To prepare whole cell lysates, cells were suspended in ice-cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.1% [w/v] SDS, 1% deoxycholic acid, and cOmplete Protease Inhibitor Cocktail), and incubated for 10 min at 100°C. Lysates were centrifuged at 15,000 rpm for 10 min, and the resulting supernatants were used as whole cell lysates. The proteins were resolved by SDS-PAGE and then transferred to a PVDF membrane. Membranes were blocked with 5% (w/v) skim milk powder in TBST buffer (20 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) for 1 h. After extensive washing with TBST, membranes were incubated with diluted primary Ab in TBST containing 1% (w/v) skim milk powder for 1 h. To detect phosphorylated protein, membranes were incubated with diluted Ab in TBST containing 5% (w/v) BSA at 4°C with gentle shaking overnight. Ab binding was detected using HRP-conjugated anti-mouse IgG or anti-rabbit IgG and visualized with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Burlington, MA) and an ImageQuant LAS4000 digital imaging system (GE Healthcare, Buckinghamshire, U.K.). When indicated, immunoblotting data were quantified by ImageJ software (National Institutes of Health, Boston, MA). Band intensities were normalized to that of the internal control (β-actin for total cell lysates and the cytoplasmic fraction; lamin A for the nuclear fraction).

Real-time quantitative RT-PCR

Total RNA was isolated from cells using TriPure Isolation Reagent (Sigma-Aldrich). The RNA samples were to reverse-transcribed using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). The quantification of mRNA expression was assessed using a TaqMan Gene Expression Assay (Thermo Fisher Scientific) for β-actin (*Actb*), hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*), IL-6 (*Il6*), IL-13 (*Il13*), CCL2 (*Ccl2*), TNF-α (*Tnf*), and IκBζ (*Nfkbiz*) or designed primer sets for *Hprt1* and CCL3 (*Ccl3*). Real-time PCR was performed using THUNDERBIRD Probe qPCR Mix or THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Reactions were analyzed in duplicate, and expression levels were normalized to *Actb* or *Hprt1* mRNA levels. The primers used in this study are shown in Supplemental Table I.

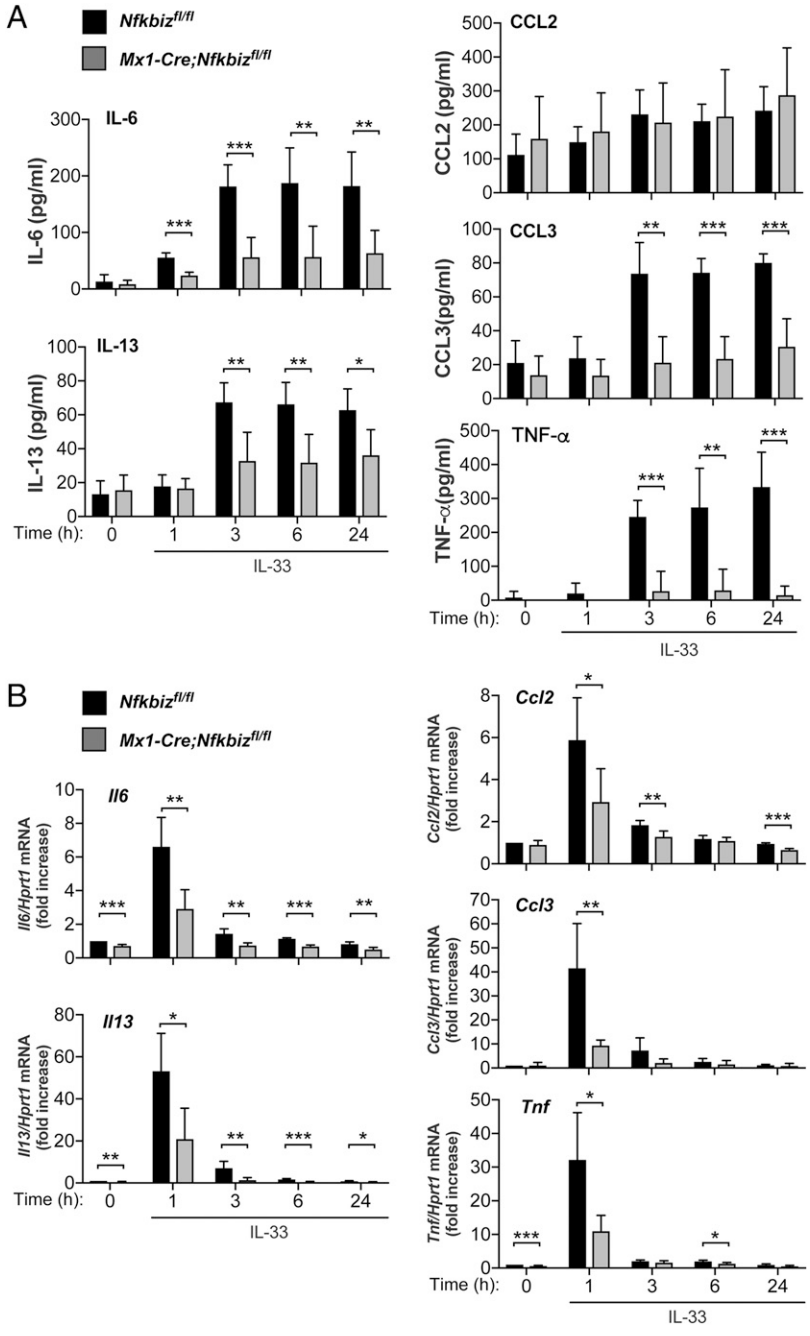

FIGURE 4. Inhibition of cytokine and chemokine production by IL-33 in IκBζ-deficient mast cells. BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) were stimulated with 3 ng/ml IL-33 for the indicated time. (**A**) Concentration of IL-6, IL-13, CCL2, CCL3, and TNF-α in the supernatant were measured by ELISA (black bar, *Nfkbiz*$^{fl/fl}$; gray bar, *Mx1-Cre;Nfkbiz*$^{fl/fl}$). Values are the mean ± SD ($n = 5$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**B**) mRNA expression of *Il6*, *Il13*, *Ccl2*, *Ccl3*, and *Tnf* were assessed using real-time RT-PCR and expressed as fold increase versus each mRNA/*Hprt1* ratio (black bar, *Nfkbiz*$^{fl/fl}$; gray bar, *Mx1-Cre;Nfkbiz*$^{fl/fl}$). Values are the means ± SD ($n = 5$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Determination of the relative levels of secreted cytokines and chemokines

Bone marrow–derived mast cells (BMMCs) were stimulated with or without 3 ng/ml IL-33 for 3 h. The relative changes in secreted cytokine and chemokine concentrations were measured using a Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems), according to the manufacturer's instructions.

Flow cytometry

Cells were blocked with purified anti-mouse CD16/CD32 Ab-containing stain buffer (PBS containing 3% FBS) and incubated with the appropriate fluorophore-conjugated Abs for 15 min at 4°C. After washing to remove unbound Abs, cells were resuspended with stain buffer containing 7-aminoactinomycin D. The surface expression of target proteins was analyzed on an LSRFortessa (BD Biosciences, Franklin Lakes, NJ). FCS files were obtained using Diva software and reanalyzed with FlowJo software (BD Bioscience). The cells were gated by side light scatter and forward light scatter, and dead cells were removed based on 7-aminoactinomycin D staining.

Isolation and culture of peritoneal mast cells

Peritoneal mast cells were isolated and cultured according to a previously described method with some modifications (20). Briefly, anesthetized mice were sacrificed by CO_2 inhalation. The cell-containing peritoneal fluid was collected by injection of 10 ml of PBS and 1 ml of air into the peritoneal cavity. After centrifugation, the peritoneal cells were resuspended and cultured in RPMI 1640 with 10% FBS, 0.05 mM 2-ME, 2 mM L-glutamine, 10 ng/ml IL-3, and 30 ng/ml mouse recombinant stem cell factor. After removing the adherent cells at day 3, nonadherent cells were collected and cultured in fresh culture medium. On day 19–20, we confirmed the expressions of c-Kit and FcεRI by flow cytometry, and the peritoneal mast cells were used for further experiments.

Statistical analysis

All data are presented as means ± SD. Statistical significance was determined using two-tailed Student *t* tests. A p value < 0.05 was considered to be statistically significant.

Results

Induction of IκBζ by IL-33 in mast cells

IκBζ is a nuclear transcription factor that regulates secondary cytokine response genes, such as *Il6* and *Ccl2*, in macrophages via TLR/IL-1R signaling (16, 21). Because IL-33 is a member of the IL-1 family of cytokines and is known to activate TLR/IL-1–related signaling via the IL-33R ST2L, we first examined whether IL-33 induces IκBζ expression. To address this, we employed bone marrow cells differentiated into mast cells (BMMCs), which express high levels of ST2L (7). We found that IL-33 transiently induced IκBζ expression in these cells (Fig. 1A–C). The production of both IκBζ protein and mRNA increased at 1 h and declined rapidly thereafter (Fig. 1A–C). In addition, IκBζ translocated into the nuclear fraction, as seen by the increase in nuclear expression (Supplemental Fig. 1A, 1B). A significant increase in *Nfkbiz* mRNA expression level was detected at 30 min, prior to the change in protein expression level, reaching a maximum level as early as 45–60 min, and rapidly declined thereafter (Supplemental Fig. 1C), suggesting that *Nfkbiz* mRNA is unstable and degrades rapidly, as described previously (21, 22). Pretreatment with anti-ST2 Ab to inhibit IL-33 binding to its cell surface receptor ST2L significantly inhibited the mRNA expression of *Nfkbiz* induced by IL-33 in a dose-dependent manner (Fig. 1D), suggesting that induction of IκBζ by IL-33 is a downstream signaling event stemming from the interaction between IL-33 and ST2L.

Cytokines and chemokines released from mast cells stimulated with IL-33

The release of cytokines and chemokines from activated mast cells is important for the generation of an inflammatory response

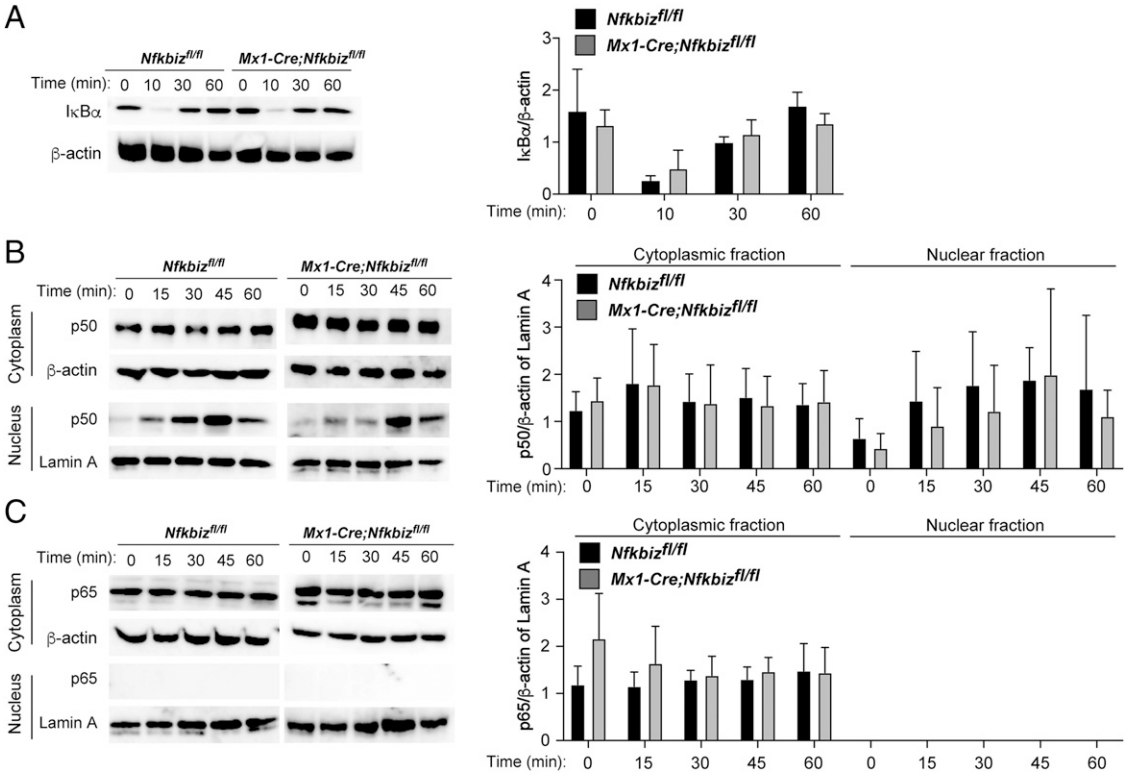

FIGURE 5. The activation of NF-κB by IL-33. BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) were stimulated with 30 ng/ml IL-33 for the indicated time. (**A**) The total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-IκBα. (**B** and **C**) Cytoplasmic and nuclear proteins were resolved by SDS-PAGE and immunoblotted with anti–NF-κB p50 (B) or NF-κB p65 (C). In the right panels, protein expression was quantified by ImageJ software. Band intensities were normalized to β-actin (cytoplasmic fraction) or lamin A expression (nuclear fraction). Data were expressed as the mean ± SD (n = 3). No significant differences between *Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$ were detected by the two-tailed Student *t* test.

(10, 12). We analyzed the release of cytokines and chemokines from BMMCs stimulated with IL-33 using a cytokine and chemokine array (Fig. 2). We set the expression cutoff levels for the cytokine and chemokine array at 5000 arbitrary units and identified five proteins that exhibited at least 2-fold increases upon IL-33 stimulation. Of the 40 cytokines and chemokines examined, the supernatant concentrations of IL-6, IL-13, CCL2, CCL3, and TNF-α were elevated by treatment with IL-33 (Fig. 2).

Generation of IκBζ-deficient mast cells

To examine the role of IκBζ in the IL-33–mediated cytokine and chemokine response, we generated IκBζ-deficient mast cells. *Nfkbiz*$^{fl/fl}$ mice were crossed with *Mx1-Cre* transgenic animals (*Mx1-Cre;Nfkbiz*$^{fl/fl}$) and the deletion of *Nfkbiz* alleles from hematopoietic cells was achieved by poly(I:C) treatment. After confirming the deletion of IκBζ in WBCs (Fig. 3A), we simultaneously differentiated bone marrow cells from *Mx1-Cre;Nfkbiz*$^{fl/fl}$ and control littermates (*Nfkbiz*$^{fl/fl}$) into BMMCs and then tested their response to IL-33. IL-33 failed to induce the expression of IκBζ in BMMCs from *Mx1-Cre;Nfkbiz*$^{fl/fl}$ (Fig. 3B–D), whereas the expression profiles of c-Kit, FcεRI, and ST2L did not differ between *Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$ (Fig. 3E, 3F).

Inhibition of IL-33–dependent cytokine production in IκBζ-deficient mast cells

We next compared cytokine and chemokine production in BMMCs obtained from *Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$ upon IL-33 stimulation. Of these five cytokines and chemokines identified, the IL-33–mediated increases in IL-6, IL-13, CCL3, and TNF-α but not CCL2 in the supernatant were significantly reduced by the deletion of IκBζ (Fig. 4A). In contrast, the mRNA expression of these cytokines and chemokines, including *Ccl2*, all increased and reached a peak 1 h after IL-33 stimulation but were significantly diminished in the IκBζ-deficient BMMCs (Fig. 4B). The deficiency of IκBζ significantly inhibited *Ccl2* mRNA but not CCL2 protein in the supernatant (Fig. 4). This discrepancy is probably because of the fact that CCL2 in the supernatant existed at high concentration, even without IL-33 stimulation, and induction of CCL2 mRNA by IL-33 was marginal (Figs. 2, 4).

The role of NF-κB, STAT5, and MAPKs in the IL-33/ST2L/IκBζ pathway

LPS-induced induction of IκBζ in macrophages is dependent on NF-κB activation (22). IL-17A and IL-36 require NF-κB and STAT3 for the induction of IκBζ in keratinocyte (23). In addition, the IL-33 signaling pathway reportedly activates p38, JNK, and ERK in mast cells, which contribute to cytokine production (24). In mast cells, IL-13 transcription is regulated, at least in part, by STAT5 activation (25, 26). Hence, we focused on the role of IκBζ for the activation of NF-κB, STAT5, and MAPKs.

To examine the role of NF-κB in the IL-33/ST2L/IκBζ pathway in BMMCs, we examined the degradation of IκBα and the translocation of NF-κB into the nucleus by IL-33. Our results indicated that deletion of IκBζ in the BMMCs did not affect the

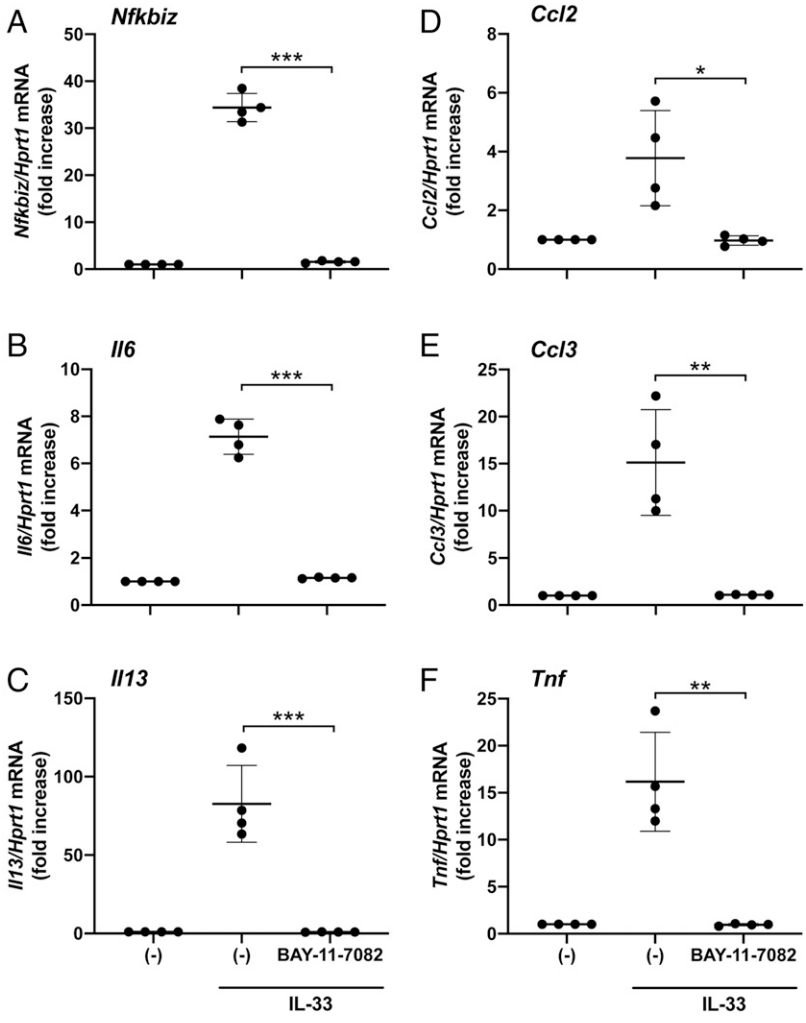

FIGURE 6. The role of NF-κB in IκBζ induction and cytokine production by IL-33. BMMCs were pretreated with DMSO or 10 μM BAY 11-7082 for 1 h and then stimulated with 3 ng/ml IL-33 for 1 h. mRNA expression of *Nfkbiz* (**A**), *Il6* (**B**), *Il13* (**C**), *Ccl2* (**D**), *Ccl3* (**E**), and *Tnf* (**F**) were assessed using real-time RT-PCR and expressed as the fold increase versus each mRNA/*Hprt1* ratio. Values are the mean ± SD ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

degradation of IκBα (Fig. 5A). In addition, IL-33 elicited the nuclear translocation of NF-κB p50 but not NF-κB p65 (Fig. 5B, 5C). This translocation of NF-κB p50 into the nucleus was not affected by the deletion of IκBζ (Fig. 5B). We next pretreated the cells with BAY 11-7082, an IκBα kinase inhibitor that inhibits degradation of IκBα. The expression of *Nfkbiz* by IL-33 was almost completely inhibited by the treatment with BAY 11-7082 (Fig. 6). Furthermore, the induction of *Il6*, *Il13*, *Ccl2*, *Ccl3*, and *Tnf* mRNAs was significantly abrogated by pretreatment with BAY 11-7082 (Fig. 6). Under the conditions employed, BAY 11-7082 at doses of up to 10 μM did not affect the cell viability assessed by trypan blue staining (Supplemental Fig. 2). These data suggest that NF-κB is critical for the expression of IκBζ and subsequent gene expression in BMMCs. Thus, IκBζ contributes to the regulation of NF-κB–induced *Il6*, *Il13*, *Ccl2*, *Ccl3*, and *Tnf* expression.

We next investigated the activation of MAPKs and STAT5. IL-33 induced the phosphorylation of p38, JNK, and ERK but not that of STAT5 in the BMMCs (Fig. 7). These signaling events were unaffected by the deletion of *Nfkbiz*$^{fl/fl}$ (Fig. 7). We further pretreated the cells with inhibitors of p38 (SB203580), JNK (SP600125), or ERK (U0126). Whereas the induction of IκBζ by IL-33 was marginally inhibited by the treatment with these inhibitors, they influenced the expression of cytokines and chemokines at various levels (Fig. 8). These data indicate that MAPKs do not mainly contribute to the induction of IκBζ, but are necessary for IL-33–induced expression of cytokines and chemokines.

Discussion

IκBζ, an atypical member of the nuclear IκB family, was originally identified as an inducible NF upon stimulation of TLRs and IL-1R (27, 28). Ligand-binding to these receptors leads to nuclear translocation of NF-κB, which directly activates a primary response gene that then stimulates protein synthesis of additional regulators that induce secondary response genes, such as *Il6* and *Lcn*, leading to signal amplification of inflammation (15). IκBζ has unique characteristics and acts as a "switch" from primary to secondary response genes to enhance cytokine production (29). Although ST2L and TLRs/IL-1R share similar cytoplasmic domains named TIRs, the roles of IκBζ in IL-33–induced responses are not fully understood. In this study, we investigated the involvement of IκBζ in the IL-33/ST2L signaling pathway axis and found that it plays a critical role in IL-33–mediated cytokine and chemokine production by BMMCs.

We observed a transient induction of IκBζ by IL-33 that led to the amplification of cytokine and chemokine production in BMMCs. To our knowledge, this is the first report to identify the importance of IκBζ in mast cells and in the IL-33–mediated signaling pathway. Mast cells are most abundant in tissues contacting the external environment, including the skin, airways, and intestines (9), where they participate in the initial host defense through their ability to secrete a variety of cytokines, chemokines, and growth factors (10). These mast cell–derived bioactive substances can, however, also exacerbate allergic responses. IL-33 is one of the strongest agonists for the production of type 2 cytokines by mast cells (24, 30) and contributes to the pathophysiology of intestinal inflammation (31), bronchial asthma, and atopic dermatitis (1). In addition, mast cell–derived cytokines exhibit diverse immune regulatory functions. For example, mast cells are required for IL-33–dependent infiltration of neutrophils, partially through the action of TNF-α (32). The IL-33/ST2L axis in mast cells is also involved in the release of inflammatory mediators, including IL-6, IL-13, CCL2/MCP-1, and PG D_2 (24, 33), which results in leukocyte recruitment and subsequent skin inflammation (34).

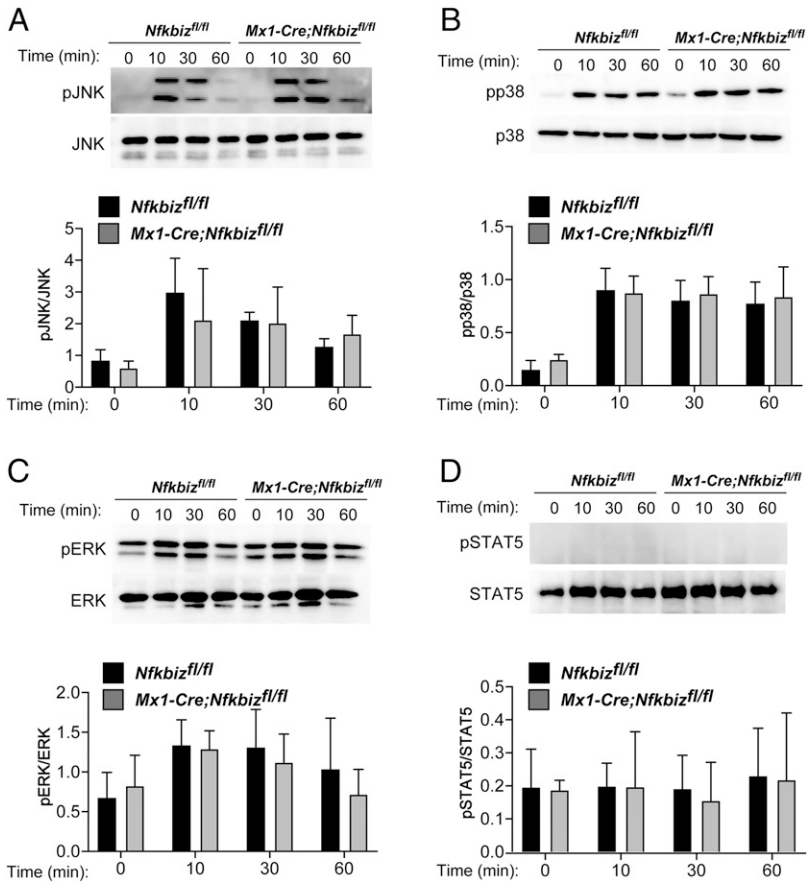

FIGURE 7. Phosphorylation of MAPKs and STAT5 by IL-33. BMMCs obtained from *Nfkbiz*$^{fl/fl}$ or *Mx1-Cre;Nfkbiz*$^{fl/fl}$ mice treated with poly(I:C) were preincubated with IL-3–depleted medium for 1 h and then stimulated with 30 ng/ml IL-33 for the indicated time. The cell lysates were resolved by SDS-PAGE and immunoblotted with anti–phospho-JNK (**A**), anti-JNK (A), anti–phospho-p38 (**B**), anti-p38 (B), anti–phospho-ERK (**C**), anti-ERK (C), anti–phospho-STAT5 (**D**), or anti-STAT5 Abs (D). In the lower panels, protein phosphorylation was quantified by ImageJ software. Band intensities were normalized to total protein expression. Data were expressed as the mean ± SD ($n = 3$). No significant differences between *Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$ were detected by the two-tailed Student *t* test.

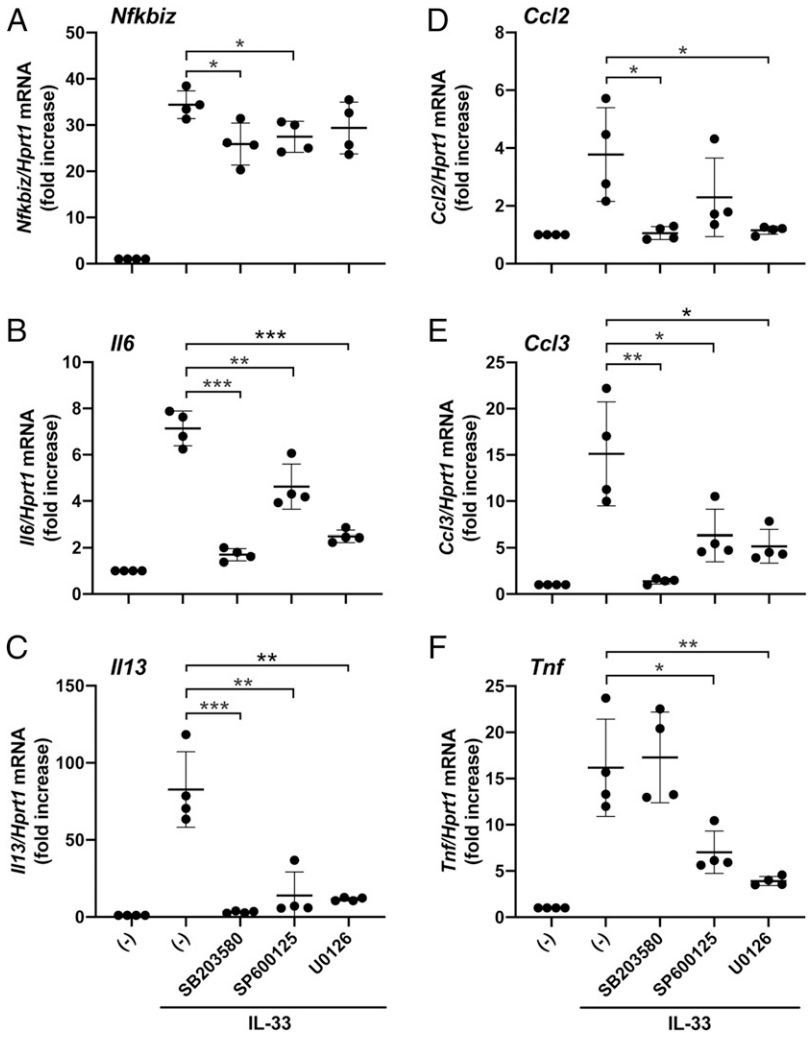

FIGURE 8. The role of MAPKs in IκBζ induction and cytokine production by IL-33. BMMCs were pretreated with DMSO or 10 μM SB203580, SP600125, or U0126 for 1 h and then stimulated with 3 ng/ml IL-33 for 1 h. mRNA expression of *Nfkbiz* (**A**), *Il6* (**B**), *Il13* (**C**), *Ccl2* (**D**), *Ccl3* (**E**), and *Tnf* (**F**) were assessed using real-time RT-PCR and expressed as fold increase versus each mRNA/*Hprt1* ratio. Values are the mean ± SD ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

Thus, targeting the IκBζ in mast cells has become an attractive approach to treat not only allergies but also a wide range of inflammatory diseases.

How does IκBζ enhance cytokine production by the IL-33/ST2L axis? IL-33 shares many components of its intracellular signaling pathway with other receptors of the IL-1 family (11). IL-33 binds to a receptor complex consisting of ST2L and IL-1RAcP, whereas IL-1R family members contain a TIR domain at their intracellular C termini that is responsible for the binding of MyD88 adaptor protein as well as IRAK1, IRAK4, and TRAF6 (13, 14). The signaling cascade results in the activation of NF-κB and MAPKs, including ERK, p38, and JNK (13, 14). The NF-κB and MAPKs signaling pathways are reportedly involved in cytokine production by mast cells stimulated with IL-33 (12). Our data suggest that IκBζ is a downstream target of NF-κB activation because an inhibitor of NF-κB completely abolished the induction of IκBζ. In contrast, the translocation of p50 into the nucleus was not influenced by the deletion of *Nfkbiz*$^{fl/fl}$, suggesting that IκBζ may be a downstream target of NF-κB, supporting NF-κB–mediated transcription in response to IL-33. In addition, activation of MAPK signaling pathways marginally affected the induction of IκBζ, but it involves in the production of cytokines and chemokines, because pretreatment with MAPK inhibitors abolished the production of a variety of cytokines and chemokines to various degrees. These data suggest that both NF-κB and MAPK pathways are involved in the initial cytokine response and that IκBζ further enhances cytokine production like a "turbocharger" to assist NF-κB's function in IL-33/ST2L signaling.

The cytokine and chemokine expression mechanisms in mast cells seems to be different from those of macrophages. Cytokine and chemokine expression in macrophages stimulated by LPS is mainly categorized into two phases (i.e., the rapidly induced primary and resulting secondary responses) (21, 29). Because IκBζ controls secondary response gene expression in macrophages, the primary response gene, such as *Tnf*, is unaffected by the deletion of IκBζ (21). In contrast, cytokine and chemokine production in mast cells stimulated by IL-33 exhibited only a single phase; all cytokines and chemokines examined were induced rapidly and simultaneously. In addition, the deletion of IκBζ diminished these responses, including TNF-α expression, in IL-33–stimulated mast cells. This discrepancy may be due to differences of the cell types or stimuli, and further experiments will be required to elucidate the mast cell– or IL-33–specific signaling pathways related to the induction of IκBζ.

IκBζ has been implicated in the pathogenesis of various diseases, including inflammation, cancer, renal pathology, and type 2 diabetes (15, 35, 36). IκBζ-deficient mice exhibit a Sjögren-like phenotype and develop severe inflammation around their eyelids with age (17). This is due to an increase in apoptosis of parotid epithelial cells, suggesting that IκBζ controls cell survival in epithelial cells. In addition, IκBζ is involved in TNF-α−, IL-17A–, and IL-36–induced expression of psoriasis-related proteins (23, 37). The expression of IκBζ is elevated in psoriatic skin, and the

phenotypes of psoriasis mouse models are completely abrogated by IκBζ knockout (37). Pretreatment with dimethyl itaconate, a novel therapeutic approach for psoriasis, alleviates the pathological conditions of psoriasis in imiquimod-treated mice by inhibiting an IκBζ-dependent pathway (38). Furthermore, several reports have suggested a role of IκBζ in cancer development. For example, the enhanced expression of IκBζ plays a role in the pathogenesis of adult T cell leukemia and B cell lymphoma (39, 40), and recurrent mutations in the 3′UTR of IκBζ dysregulate oncogene expression, leading to the activation of the NF-κB pathway (41). These data suggest that IκBζ is associated with the exacerbations of various diseases in which NF-κB is involved, and thus, it may become an attractive therapeutic target.

There are several limitations in this study that should be addressed in future work. We have not provided direct evidence for pathological roles of the IL-33/IκBζ axis in allergic diseases or for the universality of the role of the IL-33/ST2L axis in other cells expressing ST2L, including basophils, ILC2s, NK cells, and Th2 lymphocytes. We have shown that IκBζ enhances IL-33–induced cytokine and chemokine production in BMMCs. In contrast, mast cells themselves become a potent source of IL-33 that can activate ILC2 (42), suggesting that IL-33 could augment local inflammation and a Th2 response in an autocrine or paracrine manner. It will be important to elucidate the potential sources of IL-33 in each allergic disease and to show how IκBζ is involved in these processes in each cell type. Furthermore, it is possible that our results do not reflect the function of true tissue-resident mast cells because we used bone marrow mast cells differentiated ex vivo. In mice, cultured BMMCs are considered immature connective tissue-type mast cells (20). We isolated peritoneal mast cells as tissue-resident mast cells from each strain (*Nfkbiz*$^{fl/fl}$ and *Mx1-Cre;Nfkbiz*$^{fl/fl}$) and examined the mRNA expressions of *Nfkbiz*, *Il6*, and *Il13* after IL-33 stimulation. The inductions of *Nfkbiz*, *Il6*, and *Il13* were significantly inhibited in the peritoneal mast cells by *Mx1-Cre;Nfkbiz*$^{fl/f}$, as in the case with BMMCs (Supplemental Fig. 3). However, *Nfkbiz* induction could not be completely abolished in the peritoneal mast cells derived from *Mx1-Cre;Nfkbiz*$^{fl/fl}$ (Supplemental Fig. 3), suggesting that the induction of Cre by the *Mx1* promoter could not completely delete the gene in tissue-resident mast cells. To precisely clarify the role of IκBζ and IL-33 in mast cells, the use of mast cell–specific conditional deletion Mcpt5-Cre mice (43) will be required for a disease-model.

In conclusion, we have identified novel regulatory mechanisms in mast cells that enhance IL-33–dependent cytokine and chemokine production by induction of IκBζ. Our results provide the first evidence, to our knowledge, for IκBζ as an important regulator of cellular functions with respect to IL-33 signaling. Because the IL-33 and IκBζ are involved in a variety of pathophysiological conditions, targeting IκBζ may represent a novel and attractive approach for not only inflammatory diseases but also for cancer. Further studies are required to elucidate the precise mechanisms by which IκBζ controls gene expression and to apply IκBζ-targeting therapies to the treatment of a variety of diseases.

Acknowledgments

The BD LSRFortessa flow cytometer and ImageQuant LAS4000 digital imaging system were subsidized by JKA through its promotion funds from KEIRIN RACE. We thank Yaeko Suto, Mika Kishimoto, Tamaki Aoki, Nagako Sekiya, and Hiroko Hayakawa (Jichi Medical University) for technical assistance. We also acknowledge Dr. Masaki Kashiwada (Department of Biochemistry, Jichi Medical University), Dr. Shinya Watanabe, and Prof. Longzhu Cui (Division of Bacteriology, Jichi Medical University) for scientific support and technical advice. The authors thank Enago (https://www.enago.jp) for the English language review.

Disclosures

T.O. received grant support from Mitsubishi Tanabe Pharma, Novo Nordisk, Otsuka Pharmaceutical, Chugai Pharmaceutical, and Japan Blood Products Organization unrelated to this study. M.H. received a research grant from Boehringer Ingelheim unrelated to this study. The other authors have no financial conflicts of interest.

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